

Back in time: a new systematic proposal for the Bilateria

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Conventional wisdom suggests that bilateral organisms arose from ancestors that were radially, rather than bilaterally, symmetrical and, therefore, had a single body axis and no mesoderm. The two main hypotheses on how this transformation took place consider either a simple organism akin to the planula larva of extant cnidarians or the acoel Platyhelminthes (planuloid–acoeloid theory), or a rather complex organism bearing several or most features of advanced coelomate bilaterians (archicoelomate theory). We report phylogenetic analyses of bilaterian metazoans using quantitative (ribosomal, nuclear and expressed sequence tag sequences) and qualitative (HOX cluster genes and microRNA sets) markers. The phylogenetic trees obtained corroborate the position of acoel and nemertodermatid flatworms as the earliest branching extant members of the Bilateria. Moreover, some acoelomate and pseudocoelomate clades appear as early branching lophotrochozoans and deuterostomes. These results strengthen the view that stem bilaterians were small, acoelomate/pseudocoelomate, benthic organisms derived from planuloid-like organisms. Because morphological and recent gene expression data suggest that cnidarians are actually bilateral, the origin of the last common bilaterian ancestor has to be put back in time earlier than the cnidarian–bilaterian split in the form of a planuloid animal. A new systematic scheme for the Bilateria that includes the Cnidaria is suggested and its main implications discussed.

Keywords: Bilateria; Cnidaria; Acoela; Nemertodermatida; molecular phylogeny; microRNA

The time will come I believe, though I shall not live to see it, when we shall have fairly true genealogical trees of each great kingdom of nature.

(Charles Darwin in a letter to Thomas Huxley, 1857)

1. INTRODUCTION

When in the last third of the twentieth century molecular taxonomists aimed to establish the phylogenetic relationships of all organisms (the Tree of Life), they began with the following two premises: first, the increase in morphological complexity along the phylogenetic tree should run parallel to and be based on an increase in genomic complexity (e.g. number of genes; Cavalier-Smith 1985; reviewed in Hahn & Wray 2002) and second, genes and proteins should have a constant, clock-like rate of change over time (Zuckerlandl & Pauling 1965; Kimura & Ohta 1973). Therefore, under a perfect molecular clock, protein and DNA sequences would result in a complete Tree of Life delineating its main cladogenetic events. Morphological characters and new genes appearing at each node could then be used to guide an understanding of the evolution of morphological characters.

Some clues emerging from molecular biology and developmental genetics in the early 1990s proved both premises to be flawed. First, lower and higher organisms encode very similar families of transcription factors and signal transduction molecules. In other words, variation in morphological complexity in metazoan evolution is probably correlated with or caused by the variation in the amount of interactions of a more or less similar set of genes. Second, the rates of change in genes and proteins proved to be anything but clock-like and vary according to the gene, protein, lineage, site and period studied (Easteal 1985; Nei & Kumar 2000).

One main consequence of these changes is the long-lasting difficulties in resolving the so-called ancient radiations, that is, cladogenetic events which occurred a long time ago and for which morphology, fossils and molecules have, so far, not provided satisfactory answers. Here, we address arguably the most important conundrum: the origin and radiation of bilateral animals (the Bilateria).

2. THE BILATERIA: BASIC FEATURES AND TWO QUESTIONS

Bilaterians include all Metazoa with bilateral symmetry either in the adult stage or, in those cases where bilateral symmetry turned to radial symmetry (e.g. echinoderms), in the larval stage. All bilaterians are triploblastic, which means the presence of a third middle layer or mesoderm, from which most organs

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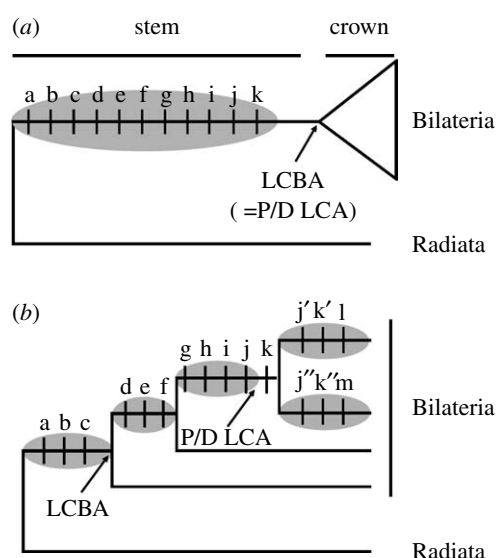


Figure 1. Coincident changes in the branch leading to the LCBA node under (a) the complex Urbilateria hypothesis (archicoelomate theory; Remane 1963; Kimmell 1996; Adoutte *et al.* 2000) and (b) the simple Urbilateria hypothesis (planuloid–acoeloid theory; Hyman 1951; Salvini-Plawen 1978). Note that in the complex Urbilateria scenario, characters (a–k) clump at the LCBA node that by definition corresponds to the last common ancestor of protostomes and deuterostomes (P/D LCA). Under the simple Urbilateria hypothesis, new clades intercalate and separate the LCBA from the P/D LCA helping to distribute character changes (a–m) across a series of stem branches and to polarize them. Under this scenario, the LCBA is morphologically simpler than the P/D LCA. Note that characters j and k could be either monophyletic (j,k) or di- or polyphyletic (j', j'', k', k''). l and m represent protostome- and deuterostome-specific characters. Grey ovals indicate the stem branches where key innovations (new characters) appeared.

form; so, true organs arise only in the triploblasts. Finally, many bilateral animals show a concentration of sensory structures and nerve cells at the anterior end of the body (e.g. cephalization). These features are widely considered basic apomorphies for the Bilateria. However, two questions remain. First, did the first bilaterian bear just this basic set of characters or, as some theories and hypotheses suggest (see below), did they also feature other characters (e.g. true brain, through gut, excretory system, body cavities (coelom), segments and even appendages and simple hearts and eyes) making them rather complex organisms? Second, do some non-bilaterian clades, traditionally considered radially or biradially symmetric (e.g. cnidarians), exhibit bilateral features and, hence, should they be considered true bilaterians?

The scores of theories advanced since Haeckel's gastraea theory on the nature of the first bilaterian (for recent updates see Willmer (1990) and Holland (2003)) could be separated into two main groups. The archicoelomate theories contemplate basic bilaterian traits such as bilaterality (hence a D–V axis) and mesoderm appearing concurrently with advanced characters such as coelom and segments. Therefore, the first bilaterians were segmented and coelomate and derived from radially symmetric, non-segmented, acoelomate cnidarians, either under larval or adult

appearance (Remane 1963; Holland 2003). Under this hypothesis, the last common bilaterian ancestor (LCBA) appears as a rather complex organism (dubbed complex Urbilateria; De Robertis & Sasai 1996; Kimmell 1996; Carroll *et al.* 2001) and is defined as the last common ancestor of Protostomia and Deuterostomia (hence, the P/D LCA; for a clarifying terminology, see Valentine (2006)). The alternative group of theories feature a more gradual scenario starting from sexually reproducing pelagic organisms (protoplanula or archiplanula), akin to present-day cnidarian planula larvae, already exhibiting some bilateral symmetry (see Salvini-Plawen (1978) for a thorough review). Under this scenario, the LCBA was a morphologically simple organism and the P/D LCA would be relegated to an internal node within the Bilateria. From this simple LCBA originated the cnidarian polyps, which settled on the substratum, as well as a stock of acoelomate, non-segmented, early bilaterians vaguely similar to present-day acoel and nemertodermatid flatworms (planuloid–acoeloid theory). From the latter stock, other acoelomates as well as pseudocoelomate and coelomate, segmented and non-segmented protostomes and deuterostomes gradually evolved.

In terms of character changes necessary between ancestors and descendants, the phylogenetic consequences of these conflicting scenarios are very different. Under the archicoelomate scenario, the number of coincident characters present at the LCBA (=P/D LCA) node is large (figure 1a). This makes it difficult to place them into any temporal order along the stem leading to the LCBA. It also implies either a large number of extinctions of intermediary taxa (stem ancestors) or a wholesale correlated transformation from one life form (radial) to another (bilateral). In contrast, the planuloid–acoeloid scenario posits a reduced number of characters at the stem leading to the LCBA and features fewer and simpler stem ancestors, a simple LCBA and a later origin for the P/D LCA (figure 1b). Hopefully, and under both scenarios, phylogenetic advances may uncover fossil or extant clades that break coincident character changes at the stem. The intercalation of these new clades will distribute inferred character changes across a series of branches instead of having them solely at the LCBA node (Donoghue 2005; Valentine 2006).

As regards whether clades outside the Bilateria do exhibit bilateral symmetry, recent genomic and gene expression analyses have shown that besides genes involved in A–P polarity, gastrulation, endodermal and germ cell specifications, cnidarian anthozoans have numerous orthologues of bilaterian gene families previously thought to be absent in 'radial' organisms (for specific references see Finnerty *et al.* 2004; Martindale *et al.* 2004; Martindale 2005; Matus *et al.* 2006a; Rentzsch *et al.* 2006). Importantly, the presence and expression in cnidarians of many of the genes involved in D–V patterning in bilaterians match ideas (going back to Stephenson (1926) and held by Hyman (1951) and Salvini-Plawen (1978)) of a second or directive axis in cnidarians (specifically in anthozoans), perpendicular to the oral–aboral (O–AB) axis (Finnerty *et al.* 2004). Were it so, cnidarians and

bilaterians might have evolved from an already bilateral ancestor, putting the origin of the bilaterians even further back in time.

3. CURRENT APPROACHES TO UNRAVEL THE ORIGIN AND EVOLUTION OF BILATERIANS

To establish whether the first bilaterians had the basic or an expanded set of embryological/morphological key characters or novelties (see above), two main approaches are currently used: (i) molecular phylogenies to sample taxa as close as possible on either side of the origin of evolutionary novelties and (ii) comparing the expression patterns of homologous genes related to these novelties among bilaterians and non-bilaterians as a criterion of homology of the corresponding anatomical structures.

Building molecular phylogenetic trees under rigorous phylogenetic inference methods aims to identify potential earliest branching bilaterians bearing novelties (e.g. symmetry, mesoderm, through gut, nephridia, coelom, segments, etc.), derived from ancestors that did not possess such features. Extant 'non-bilaterian' or 'pre-bilaterian' metazoan groups must also be searched for to be used as appropriate outgroups. As Raff (2000) states, phylogeny provides three important kinds of information: (i) it can determine the direction in which developmental features evolve, (ii) it allows evolutionary rates to be inferred, and (iii) it allows homology statements to be formulated or, conversely, tested. Information of type (i) and (iii) is particularly important as it helps to determine the 'true' groups before and after a morphological novelty and so avoid mistaken comparisons of gene expression patterns in non-homologous features (see below).

The rationale behind comparing expression patterns of developmental control genes between closely or distantly related taxa is that if in two different species orthologous genes are expressed in a similar position, these areas or regions are considered homologous, even across phyla, and should have been present in their last common ancestor. However, attempting to infer structural homology from molecular expression is fraught with difficulties (Abouheif 1997). Some of the genes tested (namely the HOX and some D-V genes; Arendt & Nübler-Jung 1994; De Robertis & Sasai 1996) are good examples of homologous genes used across phyla in homologous patterning mechanisms that result in rather different structures, i.e. HOX genes patterning the A-P axis in arthropods and chordates. Other sets of genes whose expression in embryos was used to deduce homology of structures across phyla (i.e. *DLL/distal-less* for appendages and *PAX6/eyeless* for eye development; Panganiban *et al.* 1996; Gehring & Ikeo 1999) most likely represent homologous genes patterning non-homologous structures. Finally, because most of these genes are already expressed in cnidarians, greater care needs to be exercised when homologizing morphological structures on the basis of gene expression alone (Abouheif 1997; Wagner 2007).

Such difficulties make the use of molecular characters for reconstructing a backbone tree of the Metazoa and the Bilateria an attractive option. Thereafter, morphological and gene expression characters

can be mapped onto the tree to decorate specific nodes and branches.

4. MOLECULAR PHYLOGENY OF THE BILATERIA: NEW DATA

In the last 10 years, molecular data have greatly changed perspectives on the relationships of the Bilateria. The so-called 'new animal phylogeny' (Adoutte *et al.* 2000), initially based on 18S rDNA sequence data alone, split the Bilateria into three superphyla: Deuterostomia, Ecdysozoa and Lophotrochozoa, widely accepted today. A major result was to shift all acoelomate and pseudocoelomate groups, traditionally considered at the base of the Bilateria, into the Ecdysozoa and Lophotrochozoa (Adoutte *et al.* 2000). Further nuclear and mitochondrial markers and combined morphological-molecular studies also support these findings (Peterson & Eernisse 2001; Halanych 2004).

In all schemes concerning the early history of bilaterians, the flatworms (phylum Platyhelminthes) had a central role—their simple morphology (acoelomate, non-segmented with a blind gut) coupled with a gradualistic view of evolution made them the perfect transitional taxon from cnidarian diploblasts to bilaterian triploblasts. Their monophyly, however, has always been in dispute (Smith *et al.* 1986). The first comprehensive molecular trees of the Platyhelminthes and other bilaterian and non-bilaterian phyla using 18S rDNA sequences (Carranza *et al.* 1997; Ruiz-Trillo *et al.* 1999; Jondelius *et al.* 2002) ran contrary to morphological analysis: Platyhelminthes was polyphyletic with two of its orders, the Acoela and the Nemertodermatida, branching as early bilaterian clades while the rest of the Platyhelminthes (Catenulida + Rhabditophora) fell within the Lophotrochozoa (reviewed in Baguña & Riutort 2004a,b). Importantly, the early branching position of acoels and nemertodermatids also contradicted one of the tenets of the new animal phylogeny: the non-basal position of acoelomate organisms. Sequences of other nuclear genes (including HOX cluster genes) and mitochondrial genes (Ruiz-Trillo *et al.* 2002, 2004; Cook *et al.* 2004) corroborated this early branching position. It is important to point out that based on perceived morphological synapomorphies, Acoela and Nemertodermatida are classified as sister groups forming the taxon Acoelomorpha (Ehlers 1985). However, because in most molecular trees they branch paraphyletically (Jondelius *et al.* 2002; Ruiz-Trillo *et al.* 2004; Wallberg *et al.* 2007), the monophyletic status of the Acoelomorpha is here left open and from now on dubbed 'Acoelomorpha'.

Notwithstanding these advances, the cladogenetic events at the base of the Bilateria and among most phyla belonging to the three big superphyla remain poorly resolved. This lack of resolution, also reproduced using large datasets of genes and taxa, was thought to result from the high levels of stochastic changes along presumably closely spaced cladogenetic events such as the origin and radiation of bilaterians (Rokas & Carroll 2006). However, besides stochastic errors and short time spans, incongruent or unresolved phylogenies stem from systematic errors. These errors are due to inaccuracies of the methods used in tree

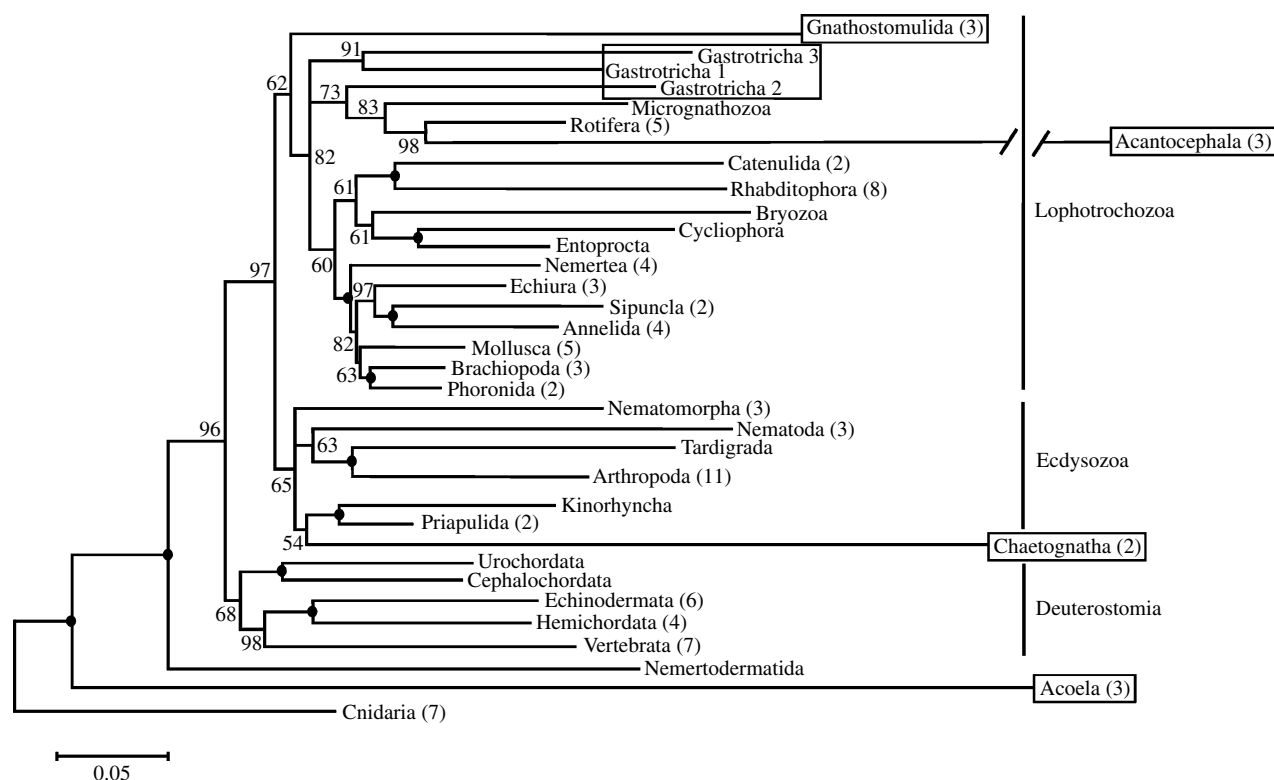


Figure 2. Bayesian analysis of 18S + 28S sequences (3696 nts) from 106 metazoan representatives with Cnidaria as the outgroup. Posterior probabilities are indicated when less than 100%, otherwise a bullet is present on the node. Phyla are collapsed and numbers in parentheses indicate the number of taxa sampled if more than one. The monophyly of each phylum has maximum support (except for gastrotrichs). Boxed groups correspond to long-branched or problematic groups for which specific analyses were carried out (see electronic supplementary material). The scale bar indicates the number of changes per site.

reconstruction directly related to model misspecifications (Felsenstein 2004). To avoid them and to improve tree reconstruction, several approaches are currently available (Philippe & Telford 2006): allowing for the numerous observed heterogeneities of the evolutionary process in models of sequence evolution (e.g. CAT model of Lartillot & Philippe (2004); Lartillot *et al.* 2007); increasing the number of taxa; removing the fastest evolving positions from the dataset; and excluding fast-evolving taxa to avoid long-branch attraction effects (LBA; Felsenstein 2004). Furthermore, the so-called rare genomic changes (RGCs; Rokas & Holland 2000; Rokas & Carroll 2006) are considered more reliable characters than conventional linear, homoplasy-sensitive sequences to avoid these problems and resolve these cladogenetic events.

In what follows, we observe these guidelines for ribosomal and nuclear gene sequences and introduce unconventional RGCs, such as microRNAs, to bilaterian phylogeny. Our main aims were to test again the position of the 'acoelomorph' flatworms as early branching bilaterians (Ruiz-Trillo *et al.* 1999, 2002) and to single out early branching phyla at the base of the three superphyla. Finally, and based on the growing consensus of cnidarians as true bilaterians (Finnerty *et al.* 2004; Martindale 2005), a new systematic proposal for the Bilateria is suggested.

(a) *Linear (quantitative) markers*

(i) *Ribosomal genes*

To minimize mutational saturation and homoplasies from ribosomal gene sequences and to avoid LBA

effects, we used methods less sensitive to LBA (maximum likelihood and Bayesian inference), model modifications such as rate heterogeneity across sites and the slowest evolving taxa available. From 564 18S and 142 28S rDNA sequences, a combined 18 + 28S rDNA dataset of over 3700 bps was obtained with 104 taxa for 28 bilaterian phyla and the outgroup. A basic dataset was obtained avoiding six long-branch (LB) phyla (Acoela, Gnathostomulida, Gastrotricha, Acanthocephala, Bryozoa and Chaetognatha) and a tree was built which reproduced the backbone of the new animal phylogeny with very high support (for further details, see electronic supplementary material). When LB phyla were introduced into this basic tree, either individually or in combination, the topology remained unchanged and statistically highly supported (figure 2; Paps *et al.*, unpublished data). Contrary to expectations, LB phyla did not cluster together at the base but fell at specific places within each superphylum: acanthocephalans with rotifers; gnathostomulids and gastrotrichs as early branching lophotrochozoans; bryozoans as sister group to a clade of cyclophorans and entoprocts; chaetognaths, albeit with very low support (bootstrap BP = 0.54), as sister group to Scalidophora; and, most importantly, a paraphyletic 'Acoelomorpha' comprising Acoela and Nemertodermatida that were, with maximum support, the earliest branching bilaterian clades.

(ii) *Other nuclear genes*

A second tree was obtained from a dataset of 13 genes (18 + 28S genes and 11 nuclear genes) from 71 species

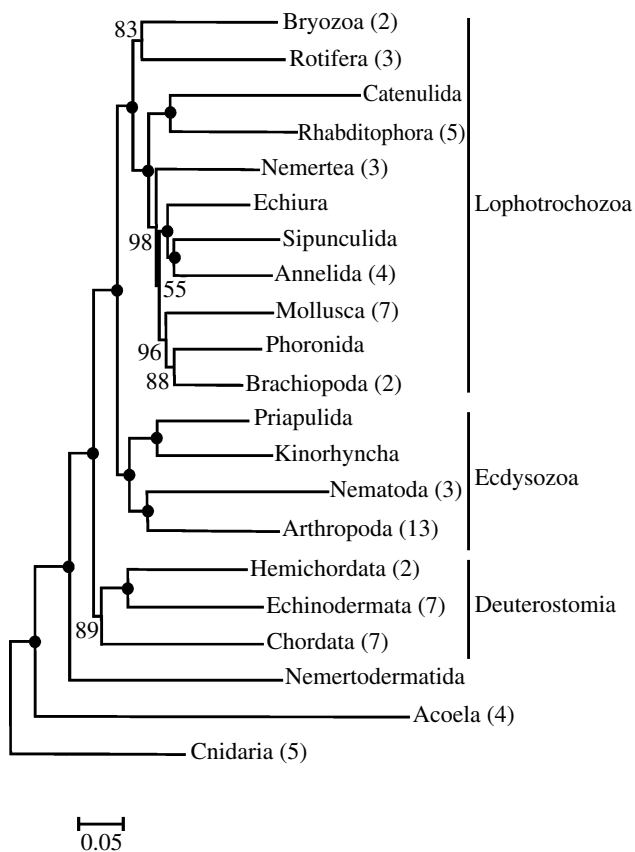


Figure 3. Bayesian analysis of concatenated sequences for 18S, 28S and 11 nuclear protein genes (9290 nts) from 74 metazoan representatives with Cnidaria as the outgroup. Posterior probabilities are indicated when less than 100%, otherwise a bullet is present on the node. Phyla are collapsed and numbers in parentheses indicate the number of taxa sampled if more than one. The monophyly of each phylum has maximum support. The scale bar indicates the number of changes per site. For further details, see electronic supplementary material.

of 21 bilaterian phyla and the outgroup. Figure 3 summarizes the tree drawn from the concatenated dataset (J. Paps, J. Baguña & M. Riutort 2007, unpublished data; see electronic supplementary material). Of the six long-branch phyla included in the 18 + 28S tree, only two (Acoela and Bryozoa) could be analysed and included here. The three superphyla appeared again with maximal support, except for a 0.89 BP value for deuterostomes. Within each superphylum, phyla grouped similarly, albeit more robustly, than in the ribosomal tree. Two differences are noteworthy: first the clustering, with low support, of Rotifera with Bryozoa (BP=0.83) and second, the low support (BP=0.55) of annelids and relatives with molluscs, phoronids and brachiopods forming, together with nemerteans, a highly supported Eutrochozoa group (BP=1.00) sister to the rhabditophoran platyhelminths. Finally, and most importantly, 'Acoelomorpha' branched paraphyletically and with maximal support at the base of the Bilateria.

(iii) Phylogenomics using expressed sequence tags

Because trees reconstructed from sequences of few genes, even from many taxa, are prone to stochastic errors while those with few taxa and many genes may generate systematic errors, gathering many sequences

from many taxa might counter both sources of errors and produce a well-resolved animal phylogeny (Philippe & Telford 2006). From several species of each phylum, 5000 expressed sequence tags (ESTs; drawn from cDNA libraries) is considered a reasonable number from which to select a set of suitable genes that, under appropriate evolutionary models, will result in better and more robust phylogenetic trees than those drawn from gene- or species-poor trees.

We produced an EST collection from the acoel *Convoluta pulchra* from which 68 different protein-coding genes were unequivocally assigned to a dataset of conserved single-copy genes from 51 species belonging to 10 different bilaterian phyla and 4 outgroup phyla. An alignment of 11 959 amino acids was established and trees inferred by maximum likelihood under the standard WAG model (Whelan & Goldman 2001) and by PHYLOBAYES analyses with the CAT mixture model that overcomes LBA artefacts when other models fail (Lartillot et al. 2007). Whereas the standard WAG model groups together the two long branches of Platyhelminthes and the acoel, the resulting tree under the CAT model, with and without the outgroup, strongly rejects this grouping. Platyhelminthes (with the exception of the acoel) fell within the lophotrochozoans. The acoel studied instead clustered either with all deuterostomes, to Xenoturbellida, or to Ambulacraria. Importantly, when the outgroup was removed, the acoel remained in a basal position, sister group to the Xenoturbellida (Philippe et al. 2007).

This is the first time using a large set of data that acoels were shown not to belong to the classical Platyhelminthes, making the latter polyphyletic. The deuterostome affinities of the acoels, however puzzling, seem to contradict the early emergence of acoels at the base of the Bilateria (Ruiz-Trillo et al. 1999). Nonetheless, the very fast evolutionary rates shown by the acoel *C. pulchra* make it not the best acoel species to study even using the CAT method. This calls for new data from slowly evolving acoels (and from its putative sister group, the Nemertodermatida) to solve this challenging phylogenetic problem.

(b) Qualitative markers

(i) HOX cluster genes

HOX and ParaHOX genes from five species of acoels and a single nemertodermatid have recently been isolated and analysed (Cook et al. 2004; Jiménez-Guri et al. 2006; Baguña et al. 2008; M. Q. Martindale 2004, personal communication). Overall, the maximal set deduced for both taxa would consist of an anterior, one/two central and one posterior HOX genes, and one representative each of the *Xlox* and *Cdx* ParaHOX genes. This putative simple HOX gene cluster in 'acoelomorpha' has been considered (Baguña & Riutort 2004a) intermediate between the expanded set (at least seven out of eight paralogy groups) of most bilaterians and the simpler set of HOX/ParaHOX genes in cnidarians (only anterior and posterior HOX and ParaHOX genes reported with no representatives of central genes; Chourrout et al. 2006; Ryan et al. 2007). Preliminary analyses of anterior, central and posterior HOX genes in the acoel *Symsagittifera roscoffensis* using BAC libraries and

fluorescent *in situ* hybridization (FISH) show them located on different chromosomes (E. Moreno, J. Baguña & P. Martínez 2007, unpublished data); therefore, the putative HOX cluster is dispersed. Preliminary results on HOX gene expression in the acoel *Convolutriloba longifissura* show a coarse collinear expression along the A–P axis (Hejnol & Martindale 2008; M. Q. Martindale 2004, personal communication). However, until whole genome sequences of acoels or nemertodermatids are made available, the presence of new, undetected, HOX and ParaHOX genes in these taxa could not be ruled out and, therefore, the precise number and type of HOX cluster genes will remain unsettled.

(ii) *microRNA sets*

The recently discovered microRNAs (miRNAs) represent new and powerful molecular markers to examine unique genetic and/or biochemical apomorphies relatively immune from homoplasy. The main phylogenetic asset is the rough correlation between the number of different miRNAs with both the hierarchy of metazoan relationships and the number of differentiated cell types as a measure of morphological complexity (Sempere *et al.* 2006). When a large set of non-paralogous miRNAs were traced along a wide range of taxa using northern blots, 21 miRNAs were found common to protostomes and deuterostomes of which none is present in sponges and two in cnidarians (Sempere *et al.* 2006). Protostomes had 12 additional specific miRNAs and deuterostomes had 7. Platyhelminthes, represented by a marine polyclad, had almost all protostome miRNAs excluding the two ecdysozoan-specific miRNAs so far detected, confirming that they are lophotrochozoan protostomes. Interestingly, the sole acoel included, *Childia* sp., had only a subset (six miRNAs) of the miRNAs shared by protostomes and deuterostomes.

Recently, we examined the miRNA complement of a second acoel taxon, *Symsagittifera roscoffensis*, and three additional rhabditophoran platyhelminth taxa, one polyclad and two triclads. *Symsagittifera roscoffensis* possesses an identical subset of miRNAs to *Childia* sp. found across protostomes and deuterostomes, and none of the miRNAs unique to protostomes or planarians (Sempere *et al.* 2007). This supports again the polyphyly of the Platyhelminthes and that the Acoela are early branching bilaterians. Were acoels members of the Platyhelminthes and simply had a reduced number of miRNAs due to secondary loss, then one would expect acoels to bear a mosaic or ‘salt-and-pepper’ pattern of miRNAs such that some primitive (triploblast specific) and some, but not all, derived (nephrozoan- and protostome-specific) miRNAs would be detected (Sempere *et al.* 2007).

(c) *Summing up*

This report and previous studies (reviewed in Baguña & Riutort 2004a) are consistent with the view that acoels and nemertodermatids are early branching bilaterian lineages (figures 2 and 3). However, two features of these figures need clarification. First, the conflict between the topology of these trees (acoels and nemertodermatids as early branching bilaterians) and trees recovered from EST analyses (acoels as

deuterostomes; Philippe *et al.* 2007). Second, the taxonomic status of acoels and nemertodermatids, either branching paraphyletically at the base (Jondelius *et al.* 2002; Ruiz-Trillo *et al.* 2004) dismissing the ‘Acoelomorpha’ as a valid taxon (Wallberg *et al.* 2007) or, as morphologists claim (Smith *et al.* 1986), sister groups forming a monophyletic Acoelomorpha.

Our EST analysis incorporated a large number of characters, though few phyla were included and acoels were represented by a single species that unfortunately had very fast clock behaviour. In contrast, ribosomal and nuclear gene analyses included fewer characters, but they had better phyla and within-phyla sampling, and acoelomorphs were represented by four out of five species (which included a nemertodermatid), some short branched. Such differences translate into striking differences in bootstrap support values as regards acoelomorph position: maximal for nuclear genes and very weak in the EST analysis (see fig. 1 in Philippe *et al.* 2007). Waiting for new data from slowly evolving acoels and nemertodermatids, current evidence and HOX cluster gene and miRNA datasets favour the topology represented in figure 4 as regards the phylogenetic position of the acoelomorphs and of the acoels in particular.

Support for a monophyletic ‘Acoelomorpha’ in molecular trees relies solely on myosin heavy chain II sequences (Ruiz-Trillo *et al.* 2002). All remaining trees and those reported here (figures 2 and 3) recover the Acoela and the Nemertodermatida as the first two separate branches within the Bilateria with high support. This dismisses the ‘Acoelomorpha’ as a valid taxon (Wallberg *et al.* 2007). How does this molecular evidence fit with claimed morphological synapomorphies linking acoels with nemertodermatids (i.e. the special structure of the basal body-rootlet system complex and the ciliary tips and the fine structure of the frontal organ; Smith *et al.* 1986)? Most of these structures occur in other metazoan groups including the Xenoturbellida, recently proposed to be a deuterostome (Bourlat *et al.* 2006). Moreover, these structures are only superficially similar and probably originated by convergence. Together with differences in sperm morphology, neurotransmitter patterns and embryonic cleavage patterns between acoels and nemertodermatids, most evidence is consistent with Acoela and Nemertodermatida as separate early branching clades and with the ‘Acoelomorpha’ as a non-monophyletic clade (Wallberg *et al.* 2007).

A second important outcome from figure 2 is the presence, albeit with moderate to low support, of acoelomate (Gnathostomulida and Gastrotricha) and pseudocoelomate (Rotifera) groups as early branching lophotrochozoans. In addition, the new Platyhelminthes (Platyhelminthes *sensu lato* excluding the acoelomorphs) either alone or with other phyla appears as sister group to the spiralian Eutrochozoa (annelids, molluscs and relatives) and not buried within the Lophotrochozoa. Together with the recent placement of the acoelomate Xenoturbellida as sister group to all deuterostomes (Perseke *et al.* 2007) or to the Ambulacraria (Bourlat *et al.* 2006), these data suggest the need to re-evaluate in depth the so-called new animal phylogeny (Adoutte *et al.* 2000).

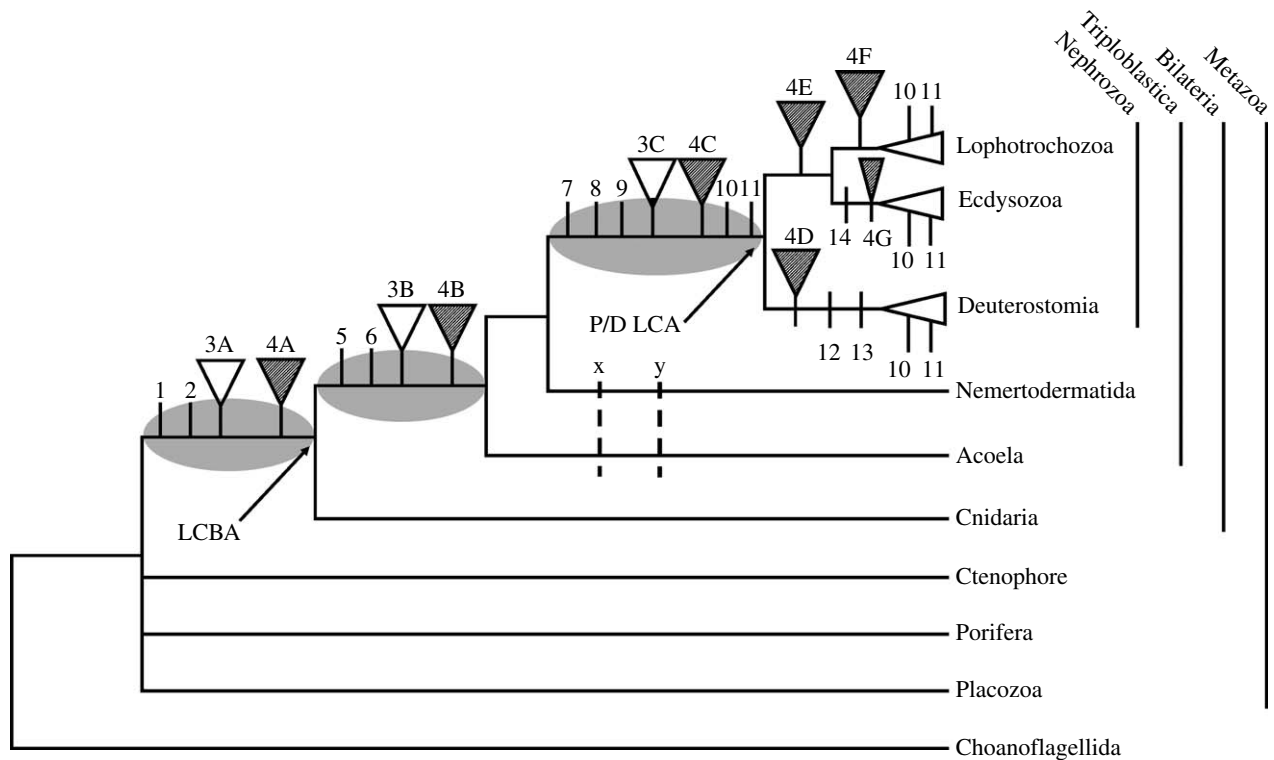


Figure 4. A new systematic proposal for the Bilateria. Morphological and molecular characters (HOX cluster genes and microRNA sets) have been mapped onto a backbone tree drawn from 18S + 28S rDNA and 11 nuclear genes. The new Bilateria includes the Cnidaria and the former Bilateria, now dubbed as Triploblastica, the latter split into a paraphyletic 'Acoelomorpha' (Acoela and Nemertodermatida) and the rest of the bilaterians or Nephrozoa. Note that the LCBA for cnidarians + Triploblastica is less complex than the ancestor for Triploblastica. Bilaterian autapomorphies (vertical solid lines and empty and hatched inverted triangles) are as follows: 1, D–V axis; 2, bilateral symmetry; 3, HOX/ParaHOX clusters (3A: 2 HOX/2 ParaHOX); 4, microRNA sets (4A: basic bilaterian set). The Triploblastica have some autapomorphies that exclude cnidarians: 3B (4 Hox/3 ParaHox); 4B, a miRNA set of five out of six genes; 5, mesoderm; 6, clustered nerve cells at the anterior end. Finally, the Nephrozoa (=Protostomes + Deuterostomes at the P/D LCA node) will have some autapomorphies that exclude acoelomorphs: 3C, an expanded HOX cluster gene of seven to eight genes; 4C, a nephrozoan miRNA set of 20 or more genes; 7, small anterior brain ganglia and ventral nerve cords; 8, through gut (mouth + anus); 9, excretory system (=protonephridia). As suggested by some authors, other autapomorphies of Nephrozoa would be: 10, coelomic cavities; 11, body segmentation, though they may have a monophyletic or a polyphyletic origin. Some autapomorphies for the Deuterostomia, Lophotrochozoa and Ecdysozoa are indicated: 4D–4G, specific miRNA sets; 12, post-anal tail; 13, gill slits; 14, ecdysis. x, y (broken lines), postulated synapomorphies for a monophyletic Acoelomorpha (Smith *et al.* 1986). x, special structure of the basal body-rootlet system complex and the ciliary tips; y, fine structure of the frontal organ. Grey ovals indicate the stem branches where key innovations appeared. See text for further details and main references.

5. THE BILATERIA: A NEW SYSTEMATIC PROPOSAL

In all zoological textbooks, cnidarians (anthozoans + medusozoans) are classified as organisms with radial symmetry. Although in most anthozoan cnidarians bilaterally symmetric features (e.g. slit-shaped mouth, internal mesenteries and asymmetric siphonoglyphs in the polyp form) were noted in the past (Stephenson 1926; Hyman 1951; Salvini-Plawen 1978; Willmer 1990), they were not taken as evidence for bilaterality because anthozoans were considered derived cnidarians and hence their internal bilateral features as secondarily evolved. Recent molecular phylogenies, however, have shown that Cnidaria and Bilateria are sister groups (Medina *et al.* 2001; Wallberg *et al.* 2004) and, importantly, that the Anthozoa is not a derived cnidarian clade but a basal group rendering its bilaterally symmetric features as possible plesiomorphies for the cnidarians (Collins 2002). Hence, cnidarians could originally be truly bilaterian (Finnerty *et al.* 2004; Martindale 2005) albeit

secondarily modified to radiality (externally) owing to their predominantly sessile life style.

The homology between the O–AB axis of cnidarians and the A–P axis of bilaterians is now widely accepted, though the precise equivalences between oral (O) and aboral (AB) ends of cnidarians to anterior (A) and posterior (P) ends of bilaterians are disputed (O = P and AB = A: Salvini-Plawen 1978; Meinhardt 2002; Rentzsch *et al.* 2007; Baguña *et al.* 2008; O = A and AB = P: Finnerty *et al.* 2004; Martindale 2005; Matus *et al.* 2006b). Moreover, the presumed homology of the 'directive axis' of cnidarians to the bilaterian D–V axis, initially based on the transient asymmetric expression of the cnidarian orthologues of *BMP2/4/dpp* and other D–V genes, has been amply corroborated by the asymmetric expressions of scores of 'endodermal', 'mesodermal' and 'neural' genes (reviewed in Martindale 2005; Matus *et al.* 2006b).

The increasing evidence of cnidarians as bilateral in origin, new molecular data (figures 2 and 3 and microRNA datasets) backing the acoelomorph

flatworms as the earliest extant branching bilaterian and the presence of acoelomate/pseudocoelomate groups at the base of the lophotrochozoans and deuterostomes prompt us to suggest a new systematic proposal for the origin and evolution of the Bilateria (figure 4). Under this proposal, Cnidaria are considered true bilaterians and the sister group to a less-inclusive bilaterian clade, here named Triploblastica, which comprises present-day bilaterians with a true mesoderm. Within the Triploblastica, molecular evidence (figures 2 and 3) favours the early branching of a paraphyletic 'Acoelomorpha' (acoels first and nemertodermatids second) sister group to the traditional protostome+deuterostome clade, or Nephrozoa (*sensu* Jondelius *et al.* 2002). Apomorphies of the new Bilateria would be the establishment and consolidation of a new D–V axis and the ensuing bilateral symmetry, and the appearance of a basic HOX cluster (2 HOX/2 ParaHOX genes) and a minimal set (two out of three genes) of miRNAs. Plesiomorphies of the new Bilateria, shared with the ctenophores, would be an A–P axis (O–AB in cnidarians and ctenophores), diploblasty (ectoderm+endoderm), the presence of muscle cells not forming a true mesoderm (Burton 2008) and the presence of a nerve net. Another key apomorphy of Triploblastica is the clustering of nerve cells at the anterior end from which longitudinal bundles of nerve fibres spring. Such characters probably run parallel to the first expansion of HOX/ParaHOX clusters (group 3 and central HOX genes; character 3B) and new miRNAs (character 4B). After acoelomorphs and other extant (Xenoturbellida) and extinct acoelomate/pseudocoelomate groups split, the radiation of Nephrozoa resulted in protonephridia, a through gut, and the progressive development of a more concentrated nervous system (layers of nerve cells surrounding a neuropile and defined as ventral nerve chords). Because the last scenario led to the appearance of true organs and more elaborate A–P and D–V axial patterns, full sets of HOX cluster genes (character 3C) and new sets of miRNAs (characters 4C–4G) were also required.

The name Planulozoa was recently proposed by Wallberg *et al.* (2004) to define a clade comprising Cnidaria and Bilateria. As such, Planulozoa is formally equivalent to the name Bilateria, here proposed for the more inclusive clade (Cnidaria+Triploblastica). Suggested synapomorphies for the Planulozoa are the presence of endodermal myoepithelial musculature, septate junctions in epithelial cells, symmetrically arranged spermatozoon heads with a mid-piece and a set of several clustered HOX genes (Wallberg *et al.* 2004). Such features, however, are plesiomorphic or have not been tested in other phyla, and, when referring to HOX clustering in cnidarians, seem at odds with most recent genomic data (Chourrout *et al.* 2006; Ryan *et al.* 2007). Moreover, the name Planulozoa stems from the presumed similarities between the planula larva and acoel worms, both being vermiform and having an apparent polar development of the nervous system (for specific references, see Wallberg *et al.* 2004). Again such characters are weakly defined, have to be tested in other phyla and refer to a

hypothetical process contemplated in the planuloid–acoeloid theory. Instead, the new Bilateria here proposed is defined by specific descriptive characters (D–V axis, bilateral symmetry and microRNA sets).

The first asset of the proposal set forth over that contemplated in the new animal phylogeny (see figure 1 for comparison) is that key changes or innovations in bilaterian evolution are spread along several stem branches allowing character states to be polarized. In particular, it unlinks D–V axis formation from mesoderm formation: the first appearing in the last common ancestor (equal to LCBA) of Cnidarians and Triploblastica and the second originated in the LCA of Triploblastica. It has been claimed that some members of the cnidarian Medusozoa possess a mesodermal derivative, the entocodon (Seipel & Schmid 2005), and that members of both Cnidaria and Ctenophora possess striated muscle, a mesodermal derivative (Seipel & Schmid 2006). This would imply that the last common ancestor of Ctenophores and Cnidaria+ Bilateria had already been a triploblast bearing striated muscle (Martindale *et al.* 2004; Seipel & Schmid 2005; Boero *et al.* 2007). However, striated muscle in cnidarians, namely in anthozoans, is epitheliomuscular; the entocodon and the mesoderm have very different developmental origins (the first from ectoderm and the second from the endoderm); and striated muscles in ctenophores, while truly muscular, non-epithelial and derived from the endoderm, are very distinct from triploblastic striated muscles (reviewed in Burton 2008). Therefore, the more parsimonious scenario is that the LCBA in figure 4 was a diploblast and that triploblastic mesoderm, cnidarian entocodon and striated musculature in Cnidaria, Ctenophora and Triploblastica had independent origins. Under this scenario, it could be predicted that genes involved in mesodermal patterning and differentiation in triploblasts (i.e. *snail*, *twist*, *forkhead*, *brachyury*, *mef2* and *GATA*) are primarily linked with the endoderm in diploblasts, and that patterning genes involved in muscle development within each lineage and in the formation of the hydrozoan entocodon (i.e. *mef2*, *Id*, *msx*) bear little or no similarity in expression. Both predictions are borne out from recent molecular data (Finnerty *et al.* 2004; Martindale *et al.* 2004; Burton 2008). This strengthens the view of separate, independent origins for muscle cells in the three clades, and for the origin of mesoderm in triploblasts from the bipotential endoderm (equal to mesoendoderm) of the LCBA. Therefore, the presence of a D–V axis in the LCBA unlinks character 2 (D–V axis) from character 5 (mesoderm), pointing to cnidarians as the group of choice to analyse the origins of the D–V axis and bilateral symmetry, and to acoels and nemertodermatids to explore the origins of mesoderm and of a more centralized nervous system.

The second asset of the phylogenetic tree in figure 4 is the closer similarities between the new LCBA and the ancestor envisaged in the planuloid–acoeloid theory than between the former and the complex Urbilateria postulated in the archicoelomate theory.

Unless acoels and nemertodermatids are shown to be ancestral but simplified or just derived bilaterians, the new LCBA leads to a smooth morphological and developmental transition from a bilateral, diploblastic planuloid to a bilateral, triploblast acoeloid, and from the latter to more complex higher bilaterians. Alternatively, bilateral symmetry may have evolved under selective pressure for improved internal circulation in a cnidarian–bilaterian ancestor, inferred to be a sessile, bilaterally symmetrical animal (Finnerty 2005). Additional internal manifestations of bilateral symmetry evolved subsequently in bilaterians. As for most proposals on the origin of bilateral organisms with directive locomotion based on the enterocoel–archicoelomate hypotheses, the main stumbling blocks for its acceptance are the undefined developmental mechanisms and the uncertain functional continuity of intermediates between a sessile ancestor and a benthic crawling descendent. This makes it more plausible that, as stated in the planuloid–acoeloid theory, bilaterality first originated in small, bottom-dwelling organisms.

6. SUMMARY AND PROSPECTS

Phylogenetic analysis using molecular markers, under strict conditions to avoid stochastic and systematic errors, have corroborated the position of acoel and nemertodermatid flatworms as the earliest extant branching members of the Bilateria. This reinforces the planuloid–acoeloid theories that see stem bilaterians as stocks of small, benthic, simple organisms probably derived from planuloid-like organisms and from which present-day cnidarians also probably arose. In addition, new molecular data are helping to overcome the simple subdivision of Bilateria into the three large and poorly internally resolved superclades introducing ‘minor’ phyla (e.g. Gnathostomulida, Gastrotricha, Chaetognatha, Xenoturbellida) into new positions that will force a re-evaluation of the new animal phylogeny (Adoutte *et al.* 2000). Finally, and most importantly, the growing consensus to consider the Cnidaria bilaterally symmetric in origin (Finnerty *et al.* 2004; Martindale 2005) leads us to suggest a new systematics for the Bilateria, which considers the Cnidaria as bilaterians and sister group to the rest of Bilateria, now dubbed as Triploblastica to indicate the appearance of mesoderm as one of the most important events in animal evolution.

In the upcoming years, refinements in data acquisition, evolutionary models, fossil record, molecular phylogenies, gene expression data (see expression of developmental genes in embryos of acoels, Hejnol & Martindale 2008) and functional evo–devo studies will be instrumental to test the soundness of the new proposal as well as to sort out the sequential evolution of clades at the base of the Deuterostomia, the Ecdysozoa and the Lophotrochozoa.

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